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EVIDENCE FOR THE EXISTENCE OF AT LEAST TWO DIFFERENT BINDING SITES FOR 5HT-REUPTAKE INHIBITORS WITHIN THE 5HT-REUPTAKE SYSTEM FROM HUMAN PLATELETS

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Abstract—Chemical modification procedures have been used to study the interaction of tricyclic and non-tricyclic 5HT-reuptake inhibitors with the [³H]imipramine binding site (IBS). *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) induced a pronounced loss in [³H]imipramine binding due to a reduction in B_{max} . Preincubation with reuptake inhibitors and subsequent inactivation by EEDQ revealed that imipramine and 5HT prevented the EEDQ-induced inhibition, but citalopram and fluoxetine did not. Thiol modification studies demonstrated that reduction by dithiothreitol (DTT) enhanced the binding of [³H]imipramine by increasing the B_{max} . The thioselective reagents 1,1-diazobis- (*N,N*-dimethylformamide) (diamide), phenyl-arsineoxide (PAO) and *N*-ethylmaleimide (NEM) attenuated the binding capacity by lowering the B_{max} . PAO, a reversible thiol reagent, prevented NEM alkylation indicating that dithiols are involved in the NEM-induced inactivation. Binding of tricyclics or non-tricyclics prior to PAO inactivation revealed that tricyclics provide complete protection against thiol modification, while the non-tricyclics do not. The results support the hypothesis that the 5HT-reuptake system of human platelets possesses at least two distinguishable binding sites.

The 5HT reuptake system appears to be an aggregate of closely interacting subunits. In spite of many binding studies (for a review see [1]) considerable uncertainty remains concerning the nature and number of inhibitor binding sites. Radiation inactivation data showed that the functional molecular size of the [³H]imipramine binding site (IBS)§ differed significantly from that of [³H]paroxetine, a non-tricyclic compound [2]. This suggests separate sites on different subunits. Reuptake inhibitors have been divided into at least two distinct groups on the basis of the cooperativity of [³H]imipramine binding [3–5]. Thermodynamic studies were compatible with this finding; non-tricyclics induced a more drastic conformational change than tricyclics [5].

Chemical modification studies can provide an additional tool for investigating the characteristics of a site at a molecular level in a non-purified system. This technique involves selective alterations of

amino-acids within or close to the binding site and, in addition, protection against modifications by prior binding of substrates or drugs. Selective reagents like dithiothreitol (DTT), 5,5'-dithio bis(2-nitrobenzoate) (DTNB), *N*-ethylmaleimide (NEM) and mercury compounds like *p*-chloromercuribenzoate (PCMB) have been used extensively to probe the potential role of sulfhydryl groups in the process of drug binding to several receptor systems, including the 5-HT reuptake system [7, 8]. Peterson and Bartfai [9] proposed that the latter might contain Cd²⁺-sensitive groups, probably vicinal dithiols. Davis [10] also investigated the involvement of the cysteines on the structure of the imipramine binding site (IBS) and the potency of reuptake inhibitors to protect against disulfide reduction by dithioerythreitol. Biassoni and Vaccari [11] reported that thiol-reducing reagents seemed to increase both the [³H]imipramine binding and the uptake of 5-HT. Furthermore, modification of the cysteine groups with NEM, PCMB and DTNB decreased the binding of [³H]imipramine.

A chemical modification approach frequently applied in *in-vivo* receptor research, involves cross-linking with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ). This reagent links carbonyl moieties to vicinal amino groups. The chemical modification studies reported below strongly favour the involvement of dithiols in the process of drug binding, and the existence of at least two distinct

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§ Abbreviations used: diamide, 1,1-azobis(*N,N*-dimethyl formamide); DTE, dithioerythreitol; DTNB, dithio (bis(*p*-nitrobenzoate)); DTT, dithiothreitol; EEDQ, *N*-ethoxycarbonyl-3-ethoxy-1,2 dihydroquinoline; IBS, imipramine binding site; NEM, *N*-ethylmaleimide; PEI, polyethylene imine; PMSF, phenylmethyl sulfonylfluoride; 5-HT, 5-hydroxy tryptamine.

binding sites: one for the so-called tricyclics, the other for non-tricyclic compounds. Modification experiments with EEDQ support the assumption of different binding sites within the 5HT-reuptake system.

MATERIALS AND METHODS

Platelet membrane preparation

The platelet membranes were prepared according to a modified version of the method of Møllerup *et al.* [12]. Outdated human platelet-rich concentrates (obtained from the Blood Bank, Groningen) were centrifuged (600 g, 10 min) to remove the erythrocytes and lymphocytes. From this point on the tissue was kept at 4° throughout the preparation. The supernatant was centrifuged (5000 g, 10 min); the pellet was washed with buffer A, 150 mM NaCl, 50 mM Tris/HCl, 20 mM EDTA, 1 mM DTT, 0.1 mM phenylmethyl sulfonylfluoride (a protease inhibitor), pH = 7.5, and resuspended in buffer B, 5 mM Tris/HCl, 5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, pH = 7.5. The platelets were lysed by homogenization of the suspension in hypotonic buffer with an Ultra Turrax (3 × 10 sec; 2000 rpm). Then the suspension was centrifuged at 50,000 g for 10 min, resuspended in buffer C (70 mM Tris/HCl, 1.0 mM DTT, 0.1 mM PMSF, pH = 7.5) and recentrifuged. Finally, the platelet membranes were resuspended in incubation buffer, 120 mM NaCl, 50 mM Tris/HCl, 5 mM KCl, 1 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 0.1 mM PMSF, pH = 7.5 and stored in liquid nitrogen at a protein concentration of 15–20 mg/ml.

[³H]imipramine binding assay

Radioligand-binding assays were performed in borosilicate culture tubes (12 × 75 mm; Kimble, U.S.A.). The specific binding of [³H]imipramine (spec. act.: 45–51 Ci/mmol) on platelet membranes was determined as the difference of binding in the absence and presence of excess citalopram (10 μM), a highly selective 5-HT reuptake inhibitor, by a filtration procedure over Whatman GF/F glass fibre filters. In short, 300 μl aliquots containing approximately 1 mg protein/ml and a fixed concentration of [³H]imipramine were incubated for 60 min at 4°. In the case of a saturation experiment, the [³H]imipramine concentration ranged from 0.3 to 20 nM. Displacement studies were performed at a fixed concentration of [³H]imipramine, in the presence of unlabelled reuptake inhibitor, ranging in concentration from 0.1 nM to 10 μM. The incubation was terminated by rapid filtration on a Millipore manifold under vacuum, using Whatman GF/F filters, preincubated in buffer containing 0.3% polyethyleneimine to reduce non-specific binding.

The filters were washed with 3 × 3 ml ice-cold buffer. Radioactivity was measured after overnight incubation in 4 ml Packard Emulsifier Scintillator 299 in a Beckman scintillation counter with a counting efficiency of about 45%.

Thiol modification studies

Inactivation with thiol reagents. Receptor-modifying reagents were added to the platelet membrane suspension after complete reduction of the receptor

suspension with 25 mM DTT for 30 min at 20° and three wash-steps with incubation buffer to reduce the DTT concentration to 1 mM. Thiol modification was achieved by reaction for 30 min at 20° with 7 mM NEM, 1 mM phenylarsine oxide (PAO) or 5 mM diamide. NEM and PAO were added from stock solutions containing about 1 M and 100 mM reagent in ethanol, respectively. The final concentration of ethanol never exceeded 1%. The NEM-induced alkylation was stopped by adding 25 mM DTT to the membrane suspension. The reversibility of the PAO- and diamide-induced oxidation was tested by incubation with 25 mM DTT for 30 min at 4° after the inactivation procedure. The binding of [³H]imipramine to the chemically modified membrane-bound IBS was determined (in quadruplicate) at a fixed [³H]imipramine concentration.

Protection against chemical modification by thiol reagents. The membrane suspension was pretreated with 25 mM DTT at 20° for 15 min, then washed to reduce the DTT concentration to 1 mM and subsequently preincubated for 30 min at 0° with excess reuptake inhibitors at the following concentrations: 10 μM for citalopram, fluoxetine, femoxetine, imipramine, clomipramine, cyanopramine and amitryptiline and 100 μM for zimelidine, nor-zimelidine and 5HT. Then the suspensions were exposed to 1 mM PAO for 30 min at 20° as described above. After the incubation procedure, the membrane suspension was rinsed at least three times with incubation buffer by centrifugation at 50,000 g for 10 min to remove the reuptake inhibitor. To test the completeness of the washing-procedure, a so-called washing control was included (preincubation with reuptake inhibitors followed by the above mentioned wash step). Finally the [³H]imipramine-binding was determined at 5–7 nM, in quadruplicate.

Inactivation with EEDQ

The optimal inactivation conditions were determined by examining the effects of incubation time at 20°, pH = 7.0 and 0.4 mM EEDQ, the effects of EEDQ-concentration at 20°, pH = 7.0 for 30 min and the effects of incubation pH at 20°, 0.4 mM EEDQ for 30 min. The inactivation was started by adding EEDQ, dissolved in ethanol, to the receptor suspension in 50 mM sodium phosphate, 70 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM EDTA. The inactivation was stopped with 10 mM DTT, putting the suspension on ice. Then the suspension was rinsed once with incubation buffer to reduce the EEDQ concentration. The [³H]imipramine-binding was determined at fixed [³H]imipramine concentration (5–7 nM). Protection against modification of the IBS with reuptake inhibitors was performed as follows. The membrane suspension, in the sodium phosphate buffer, was preincubated with excess reuptake inhibitor (1 μM imipramine, fluoxetine, citalopram or 100 μM 5HT) for 30 min at 4°. Then the suspension was treated with 0.4 mM EEDQ at 20° and pH = 6.5 for 30 min. The inactivation was terminated with addition of 10 mM DTT putting the suspension on ice and the samples were washed three times with incubation buffer to remove excess reuptake inhibitor. Finally, a [³H]imipramine saturation curve was determined (in triplicate).

Data calculation and statistical analysis

The saturation curve data were subjected to Hanes-Woolf analysis [13] or to a non-linear fitting program (minimizing the sum of squares via the Simplex-iteration procedure). The pseudo-Hill coefficients were calculated from displacement curves using a computerised non-linear regression procedure. Data were evaluated statistically using the Student's *t*-test for paired values.

Materials

All chemicals were reagent grade. The reuptake inhibitors were generous gifts from their respective firms: imipramine and clomipramine from Ciba-Geigy Lab (Basel, Switzerland), cyanopramine and amitriptyline from Roche (Neuilly-sur-Seine, France), fluoxetine from Lilly Co. (Indianapolis, IN), citalopram from Lundbeck (Copenhagen, Denmark), norzimelidine and zimelidine from Astra (Sweden) and finally femoxetine from Ferrosan (Denmark). [³H]imipramine was purchased from New England Nuclear (Boston, MA; 45.3 Ci/mmol). Outdated platelet-rich plasma was kindly supplied by the Bloodbank, Groningen, The Netherlands.

RESULTS

Displacement studies on the IBS

The binding of radiolabelled ligand has been monitored in the presence of unlabelled ligand, varying in concentration from 0.1 nM to 10 μ M. From these studies, the apparent inhibition constant, K_i , and the pseudo Hill coefficient could be evaluated. These displacement studies were done to determine the pharmacological profile of the 5HT-reuptake system. Analysis of the results revealed that tricyclics have a pseudo-Hill coefficient of one, suggesting normal competitive displacement, whereas the non-tricyclics tested in these experiments possessed a pseudo-Hill coefficient significantly smaller than 1, indicative of negative cooperativity (Table 1; $P < 0.01$). These intriguing data prompted us to investigate the interaction of tricyclics and non-tricyclics with the 5HT-reuptake receptor more thoroughly.

EEDQ-induced inactivation of [³H] imipramine binding

The kinetics of EEDQ-induced crosslinking are displayed in Fig. 1. The control sample (without EEDQ) did not change significantly during the exposure time. However, the exposed samples did

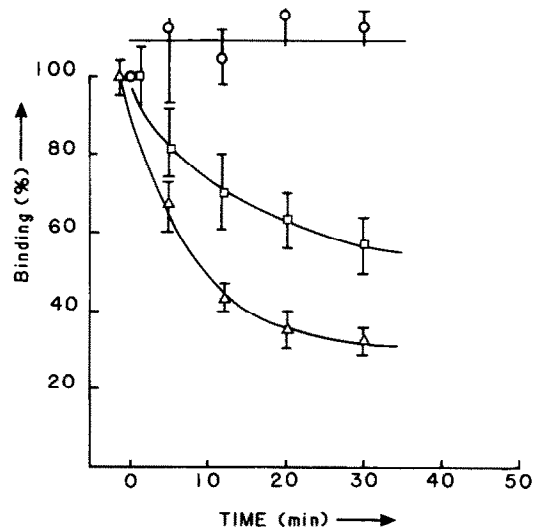


Fig. 1. Effect of the incubation time with EEDQ on [³H]imipramine binding. Incubations were done at 0.1 mM EEDQ (\square), 0.8 mM EEDQ (\triangle) and in the absence of EEDQ (\circ) at 20°, pH = 7.0. Binding is shown as the % of specific binding (mean \pm SEM of quadruplicate measurement) relative to a non-exposed control sample.

exhibit an inactivation of binding capacity. The inactivation levelled off at 40%. This was most likely a consequence of limiting EEDQ concentrations. The inactivation rate obeyed simple first order kinetics with half-times of 6 min and 30 min at EEDQ concentrations of 0.8 and 0.1 mM, respectively. A strong pH dependence was observed. No inactivation occurred at high pH but a marked increase in inactivation was observed with decreasing pH (Fig. 2). Consequently, a pH of 6.5 was selected for inactivation and protection studies.

The potency of some reuptake blockers to protect against EEDQ-induced crosslinking was determined as follows. The membrane suspension was pre-incubated with 1 μ M inhibitor (100 μ M in the case of 5HT on account of its low affinity) prior to EEDQ-induced inactivation. After treatment of the membrane suspension with EEDQ, as described in Methods, the modification was terminated by addition of DTT. The membrane suspension was rinsed thoroughly to remove the drug. Then saturation curves of the various suspensions were determined. Evaluation of the saturation curve data

Table 1. Pseudo Hill-coefficients of several 5HT-reuptake inhibitors determined from displacement of [³H]imipramine

Compound	Hill-coefficient	Compound	Hill-coefficient
Imipramine	0.99 \pm 0.09 (4)	Fluoxetine	0.73 \pm 0.21 (2)
Clomipramine	1.15 \pm 0.14 (3)	Citalopram	0.60 \pm 0.03 (2)
Cyanopramine	0.92 \pm 0.16 (2)	Femoxetine	0.42 \pm 0.08 (1)
Amitriptyline	1.28 \pm 0.21 (1)	ORG 6582	0.73 \pm 0.20 (1)
5HT	0.78 \pm 0.13 (1)	Nor-zimel.	0.64 \pm 0.16 (1)

The data were evaluated using a computerised non-linear regression procedure (Graph-Pad). Hill coefficients are given as mean \pm SEM (in case of a single experiment \pm SD). In parentheses the number of experiments.

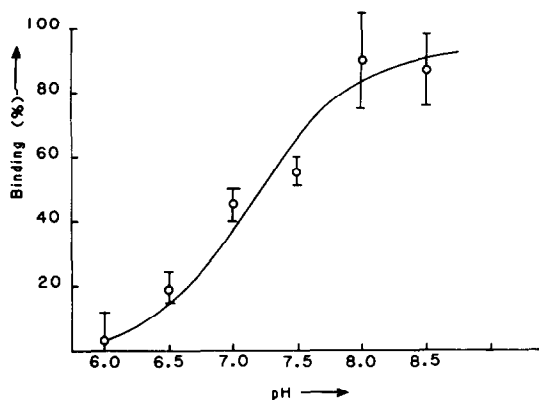


Fig. 2. Influence of the incubation pH on the inactivation of [^3H]imipramine binding by EEDQ. Inactivation was carried out with 0.4 mM EEDQ at 20° for 30 min. Binding is shown as the % of specific binding relative to a non-exposed control sample. Mean of 2 experiments.

revealed that EEDQ inactivation left the K_d unchanged ($K_d = 3.07 \pm 0.27$ nM) but lowered the B_{\max} significantly (Table 2). This reduction in B_{\max} was quite reproducible. Preincubation of the receptor with imipramine and 5HT gave partial and complete protection, respectively, against EEDQ inactivation, whereas fluoxetine and citalopram did not protect at all.

Modification of cysteine by thiol reagents

Preliminary experiments indicated that the redox state of the reuptake receptor varied from one membrane preparation to another and manifested itself in an altered B_{\max} value. Consequently the influence of oxidation and reduction on B_{\max} was investigated. As previously reported by Biassoni and Vaccari [11] and Davis [10], pretreatment with DTT had a positive effect on the binding by enhancing the B_{\max} by about 35% ($P < 0.001$; Table 3a). Incubation time and DTT concentration-dependent studies showed that maximum stimulation could be achieved by a

Table 2. Protection of the IBS against EEDQ-induced inactivation

Treatment	Binding-parameters		
	Inactivation with EEDQ	K_d (in nM)	B_{\max} (in % of control)
—	—	3.4 ± 0.4	100 ± 13
1.0 μM imipramine	—	3.7 ± 0.5	97 ± 4
—	+	$2.8 \pm 0.2^*$	$47 \pm 5^\dagger$
1.0 μM imipramine	+	2.9 ± 0.4	$69 \pm 2^*$
100 μM 5HT	+	3.7 ± 1.3	110 ± 12
1.0 μM citalopram	+	2.7 ± 0.6	$33 \pm 14^\dagger$
1.0 μM fluoxetine	+	2.2 ± 0.7	$23 \pm 2^\dagger$

The receptor suspensions were preincubated with excess reuptake inhibitor (1 μM imipramine, fluoxetine, citalopram and 100 μM 5HT) prior to inactivation at pH 6.5 and then reacted with 0.4 mM EEDQ, pH = 6.5 at 20° for 30 min. After termination of the modification with DTT (to a final conc. of 10 mM), the suspensions were washed extensively by a three-fold centrifuge step to remove the reuptake inhibitor, equilibrating the suspensions for 30 min between these wash-steps. Binding parameters from saturation curve data were evaluated via Hanes-Woolf analysis (significance: * $P < 0.01$; $^\dagger P < 0.005$ relative to control).

Table 3. Binding parameters from saturation curve data evaluated via non-linear regression

(A) Effect of reduction of the membrane suspension with 25 mM DTT (15 min at 20°) on the binding parameters (* $P < 0.001$ relative to the control value)

Sample	Binding-parameter K_d (in nM)	B_{\max} (in fmol/mg)
DTT-treated	2.8 ± 0.5	$670 \pm 7\%^*$
Non-treated	2.3 ± 0.4	$490 \pm 1\%$

(B) Effect of thiol modification by NEM (7 mM, 30 min, 20°), PAO (1 mM, 30 min, 20°) and diamide (5 mM, 30 min, 20°) after reduction of the membrane suspension with 25 mM DTT for 15 min at 20° ($^\dagger P < 0.0001$ relative to the control value)

Sample	Binding parameter K_d (in nM)	B_{\max} (in pmol/mg)
Control	1.7 ± 0.5	$453 \pm 6\%$
PAO	1.5 ± 0.5	$240 \pm 2\%^\dagger$
NEM	2.1 ± 0.6	$227 \pm 5\%^\dagger$
Diamide	1.4 ± 0.4	$187 \pm 1\%^\dagger$

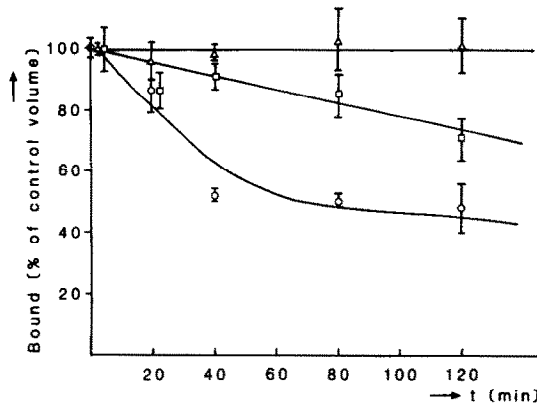


Fig. 3. Effects of DTT on $[^3\text{H}]$ imipramine binding. The membrane suspension was first reduced with 25 mM DTT for 15 min at 20° then washed to remove the DTT. Specific $[^3\text{H}]$ imipramine binding (in % of control value at $t = 0$ min) as a function of incubation time at 20° in the absence (○) or in the presence of DTT (1 mM: □; 25 mM: Δ). Mean \pm SEM of measurement in quadruplicate.

30 min incubation with 10 mM DTT at 20° (data not shown). As a result of these experiments a standard pretreatment protocol was selected involving pre-incubation with 25 mM DTT for 15 min prior to modification. Figure 3 shows that this fully reduced state of the IBS was not stable in the absence of DTT. The binding diminished to about 40% within 2 hr at 20° by reoxidation, despite the presence of EDTA. Oxidation was considerably slower in the presence of 1 mM DTT. In view of the maximum incubation time at 20° , 1 mM DTT was satisfactory to keep the redox state relatively constant.

The kinetics of modification with NEM (7 mM), diamide (5 mM) and PAO (1 mM) are described in Fig. 4. The control did not change during the

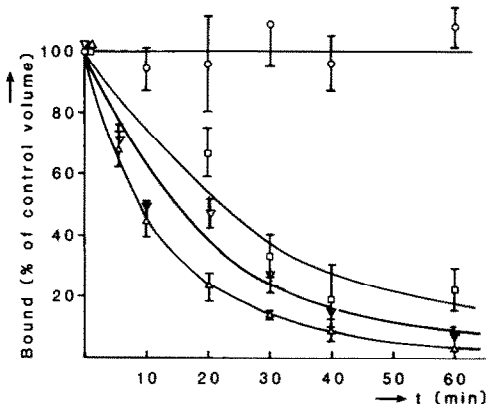
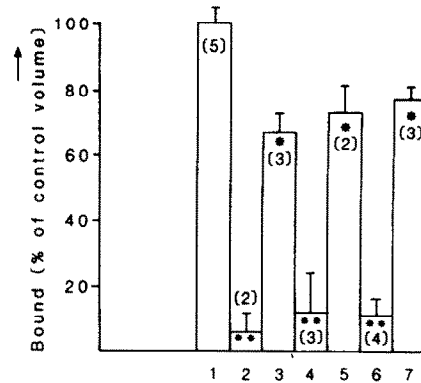


Fig. 4. Kinetics of inactivation of $[^3\text{H}]$ imipramine binding by NEM (□), PAO (▽) and diamide (Δ). After reduction of the platelet membranes with 25 mM DTT for 15 min at 20° , the DTT concentration was reduced to 1 mM by a wash step and the membrane suspension was exposed to 7 mM NEM, 1 mM PAO or 5 mM diamide or H_2O (○) at 20° . Aliquots were taken at various times and $[^3\text{H}]$ imipramine binding was determined. The specific binding is plotted on the vertical axis as the mean \pm SEM, of a quadruplicate determination. The data are plotted as the % of the control value at $t = 0$ min.



Protocol:

Protection	-	p	p	d	d	-	p
Alkylation	-	-	a	-	-	+	+
DTT-reduction	+	-	+	-	+	+	+

Fig. 5. Reversibility of inhibition by PAO or diamide and protection against NEM-induced inhibition of $[^3\text{H}]$ imipramine binding. Membranes were exposed to a protector, 1 mM PAO or 5 mM diamide, an alkylator, 7 mM NEM, and a reductant, 25 mM DTT or some combination of these reagents in the order given at the bottom of the figure. The exposure times were 30 min for PAO, diamide, NEM and for DTT. The specific $[^3\text{H}]$ imipramine binding is plotted on the vertical axis as the average \pm SEM, of the number of experiments given in the parentheses, each of which was carried out in quadruplicate. The data are plotted as the % of the non-exposed control. Significance: ** $P < 0.0001$; * $P < 0.01$ vs control.

incubation period. NEM, diamide and PAO induced a pronounced loss of $[^3\text{H}]$ imipramine binding with a $t_{1/2}$ of 22 min, 10 min and 15 min, respectively. The NEM alkylation rate is in agreement with the results obtained by Davis [10]. Saturation curves were measured to determine whether the modification influenced only the B_{max} and, consequently, whether it was permissible to determine the binding at a fixed $[^3\text{H}]$ imipramine concentration. These data are presented in Table 3b. Only the B_{max} was affected by NEM, PAO and diamide; the reduction in B_{max} achieved in this experiment ($\pm 50\%$) was less than the value obtained in the experiments described in Fig. 4 ($\pm 25\%$ after 30 min). Since the membrane suspension in the saturation-curve experiment contains about 3 times as much protein (15 mg/ml) during the inactivation with PAO as in the experiments described in Figs 4–6, the observed discrepancy might be a consequence of slightly limiting PAO concentration. Nonetheless, the affinity remained virtually unaltered. The reversibility of PAO and diamide inactivation and the ability of PAO to protect against alkylation by NEM are shown in Fig. 5. After gross reduction of the IBS with 25 mM DTT, the washed membrane suspension containing 1 mM DTT was exposed for 30 min at 20° to 1 mM PAO or 5 mM diamide. The mixture was then put on ice. Protection against NEM alkylation was tested by subsequent treatment of the suspension with 7 mM NEM for 30 min at 20° , followed by a reduction step with 10 mM DTT. Reversibility of the oxidation was determined by reduction of the PAO and diamide

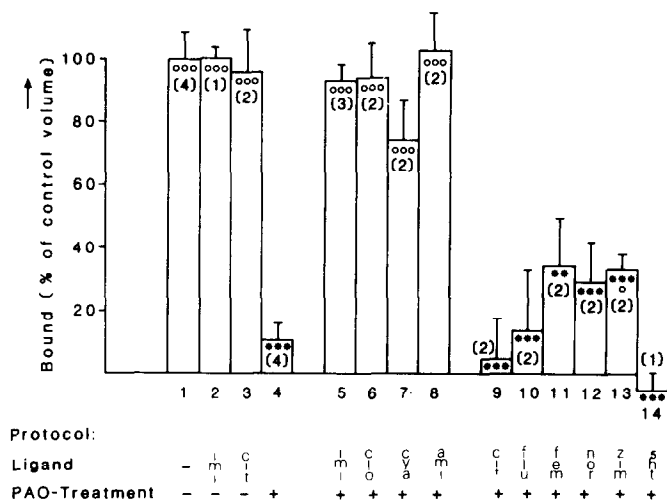


Fig. 6. Protection against PAO induced thiol modification by various reuptake inhibitors. Non-tricyclic inhibitors were: citalopram, 10 μ M; fluoxetine 10 μ M; femoxetine 10 μ M; zimelidine 100 μ M; nor-zimelidine 100 μ M and tricyclic reuptake inhibitors were: imipramine 10 μ M; cyanopramine 10 μ M; clomipramine 10 μ M; amitryptiline 10 μ M and finally the endogenous drug 5HT, 100 μ M. After reduction of the membrane suspension with 25 mM DTT (15 min, 20°) the samples were preincubated for 1 hr (4°) with reuptake inhibitors, followed by thiol modification with PAO (1 mM, 30 min, 20°). After the modification the samples were washed three times to remove the reuptake inhibitor and [3 H]imipramine binding was measured as stated in Methods. No DTT was present during the binding measurements. The specific [3 H]imipramine binding as % of non-exposed, non-preincubated control is plotted on the vertical axis. The value is the mean \pm SEM, of the number of experiments given in parentheses. Each experiment was run in quadruplicate. Significance *** $P < 0.001$ vs control, ** $P < 0.005$, * $P < 0.01$. $\circ\circ\circ$ $P < 0.001$ vs PAO-exposed sample, $\circ\circ$ $P < 0.005$, \circ $P < 0.01$. A control of the wash-step was included.

treated samples with excess DTT. Diamide, PAO, and NEM inactivate the 5HT-reuptake system to about the same extent. The diamide and PAO induced inhibition is reversible: the [3 H]imipramine binding returned to 75% of its original value. The fact that reversible reaction with PAO protected against NEM induced alkylation indicates that NEM also reacted with the same dithiol.

The main point of interest was to test whether or not the tricyclic reuptake inhibitors interact with the same binding site as do non-tricyclics. Figure 6 examines the ability of tricyclic and non-tricyclic compounds to protect against inactivation by PAO. PAO treatment resulted in a 90% decrease of [3 H]imipramine binding. This inactivation was prevented by preincubation with tricyclics ($P < 0.0001$). As expected from the results of EEDQ inhibition studies, even though nontricyclics are able to displace imipramine from the IBS, they do not protect against PAO modification. The observed inactivation after preincubation with non-tricyclics was not due to incomplete washing, since the washing control and the untreated control both showed the same high value. The only discrepancy with the EEDQ protection study lies in the paradoxical effect of 5HT. It was not capable of protecting against thiol activation. This was not due to oxidation of 5HT; however, we have monitored oxidation of 5HT during the incubation time (30 min at 20°) by following the UV spectrum of 5HT under the experimental conditions and found no significant change was observed in the UV spectrum (265–320 nm) of 5HT for at least 1 hr (data not shown).

DISCUSSION

EEDQ-induced receptor inactivation

EEDQ has been one of the inhibitors most frequently applied, *in vivo*, in order to quantify *de novo* receptor synthesis [14, 15] or to investigate the potency of drugs to protect against inactivation of their respective receptor systems [16]. However, it is not widely used in *in vitro* research, despite the obvious advantages for comparison with *in vivo* studies. We have examined the efficacy of two categories of reuptake inhibitors and 5HT to protect against receptor inactivation by EEDQ in order to clarify the possible existence of multiple binding sites within the reuptake system. Analysis of Hanes-Woolf isotherms revealed that the B_{max} decreased 53% upon EEDQ modification. The K_d was left essentially unchanged.

Reuptake inhibitors can be divided into two classes, based on their efficiency to protect against EEDQ inactivation. 5HT and imipramine protect against EEDQ-induced inactivation, whereas the non-tricyclics do not protect. In fact, the nontricyclics tend to enhance the EEDQ-induced inactivation. From these results it is difficult to decide if the protection of imipramine and 5HT against modification by EEDQ implies a direct blockade of the actual binding site or a binding induced conformational change, which attenuates the accessibility of amino/carbonyl groups involved in the inactivation.

Chemical modification of cysteines

Cysteines have been documented in many dif-

ferent receptor systems including the adrenergic [7], dopaminergic [8] and serotonergic systems [9–11, 17]. Biassoni reported that both [^3H]imipramine binding and 5-[^3H]T reuptake was stimulated by reduction of what were thought to be disulfides [11]. In agreement, alkylation or oxidation inhibited both processes. From these studies he proposed that the DTT-induced increase of the drug binding involved the low- rather than the high-affinity state of the [^3H]imipramine binding protein. Our thiol modification experiments indicate that DTT indeed has a stimulating effect on the binding of imipramine by enhancing the B_{max} 35% presumably by a decrease of the fraction of oxidized IBS. The stimulatory effect of DTT appeared to be dependent on the relevant tissue batch. This might be a consequence of minor differences during tissue preparation. Spontaneous oxidation appeared to lead to a 60% decrease in binding, whereas chemical oxidation with diamide resulted in a 90% decrease of binding capacity. This difference is most likely due to the redox potential of the incubation mixture. No effort was made, in the spontaneous oxidation experiment, to saturate the reaction mixture with O_2 . The diamide- and PAO-induced inhibition was reversible for 75–80%. Clearly, vicinal dithiols are involved in the thiol modification, which results in a reduction of [^3H]imipramine binding.

The rather slow NEM modification as observed in our laboratory and by Biassoni and Vaccari [11] and Davis [10] suggests that NEM might inactivate by alkylating an inert or hidden thiol group or by reacting non-specifically [18]. In view of the data presented above, we can exclude the latter. Either the thiols are chemically inert or not readily accessible. The observed loss in [^3H]imipramine binding activity after thiol modification could be explained as an alteration in tertiary structure or as a steric hindrance of the binding or it could implicate the thiol directly in the process of drug binding itself.

Davis [10] reported that blockade of the IBS with the non-tricyclic reuptake inhibitor fluoxetine prevented the DTE-induced increase in binding capacity. The results presented in this study are not compatible with this feature; non-tricyclics are not capable of providing any protection against thiol-modification. By contrast, tricyclics protect the IBS against both EEDQ- and PAO-induced inactivation. The Hill coefficients calculated from our displacement studies also suggested another type of inhibition of [^3H]imipramine for non-tricyclics relative to tricyclics. The Hill coefficients, obtained by Sette *et al.* [4] and Mellerup *et al.* [12] were essentially the same. In agreement with our conclusions, Reith [6] has reported that the non-tricyclics induce a more drastic conformational change than the tricyclics, possibly from a different type of interaction with the protein. Other studies support this finding [3, 4, 6]. Wennogle [19] concluded that one group of displacers accelerated, whereas a second group attenuated the dissociation rate of [^3H]imipramine.

On the grounds of the data provided by this study and by others, one could propose that binding of non-tricyclics involves a binding site, which is closely associated with but not identical to the IBS. The regulatory IBS, part of the presynaptic complex,

modulates 5HT reuptake. The nature of the coupling of the IBS and 5HT-reuptake system remains to be clarified. 5HT appears to provide protection against EEDQ-induced crosslinking but not against thiol modification. It is rather unlikely that its protective potency is caused by direct reaction with EEDQ, since the EEDQ concentration exceeds by far that of serotonin. Thiol modification might affect another part of the IBS, possibly the actual recognition site, while EEDQ might induce a drastic structural change. EEDQ-induced inactivation may be prevented by conformational changes induced by the binding of 5HT or imipramine. By contrast, binding of 5HT might leave the thiol groups, essential for imipramine binding, still accessible for thiol-modifying reagents.

Further studies on the purified 5HT reuptake system will be necessary to provide a decisive answer about the actual mechanism of 5HT reuptake and its modulation.

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